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Local Gurken signaling and dynamic MAPK activation during *Drosophila* oogenesis

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Abstract

During *Drosophila melanogaster* oogenesis Gurken, a TGF-α like protein localized close to the oocyte nucleus, activates the MAPK cascade via the *Drosophila* EGF receptor (DER). Activation of this pathway induces different cell fates in the overlying follicular epithelium, specifying the two dorsolaterally positioned respiratory appendages and the dorsalmost cells separating them. Signal-associated internalization of Gurken protein into follicle cells demonstrates that the Gurken signal is spatially restricted and of constant intensity during mid-oogenesis. At the same time MAPK activation evolves in a spatially and temporally dynamic way and resolves into a complex pattern that presages the position of the appendages. Therefore, different dorsal follicle cell fates are not determined by a Gurken morphogen gradient. Instead they are specified by secondary signal amplification and refinement processes that integrate the Gurken signal with positive and negative feedback mechanisms generated by target genes of the DER pathway.

Keywords: argos; *broad-complex; Chorion; cornichon*; Dorsoventral patterning; Dorsal appendages; Epidermal growth factor receptor; *gurken*; *K10*; *rhomboid*; *spitz*; *sprouty*; Transforming growth factor-α; *torpedo*

1. Introduction

The egg of the fruitfly *Drosophila melanogaster* is polarized along its anteroposterior and dorsoventral axes through the asymmetric localization of instructive molecules during oogenesis. Spatial fates along the dorsoventral axis can easily be recognized in mature eggs by the presence of different external chorionic structures. These structures are derived from the somatic follicular epithelium that surrounds the growing oocyte and its 15 sister germline cells that have differentiated into nurse cells. An example of such chorionic specializations are the two long respiratory appendages that are located dorsally at the anterior end of the egg. These appendages are formed by two groups of follicle cells that migrate over the anterior end of the oocyte at stage 11 of oogenesis. While these two dorsolateral groups of cells contribute to the formation of the appendages, the dorsalmost cells separating them do not, but rather contribute to more dorsal and anterior structures of the egg shell (reviewed in Spradling, 1993).

Recent work has shown that a signal emanating from the oocyte, namely Gurken, is required twice during oogenesis to induce fate changes in the follicular epithelium. During early stages of oogenesis Gurken signaling from the posteriorly localized oocyte instructs the follicle cells in contact with the oocyte to adopt a posterior fate (Gonzalez-Reyes et al., 1995; Gonzalez-Reyes and St. Johnston, 1998; Roth et al., 1995). Later, as the germinal vesicle assumes an asymmetric position at the anterior cortex of the oocyte, *gurken* (*grk*) mRNA and protein become tightly localized to its vicinity. Gurken signaling will then induce dorsal fates in the adjacent follicle cells. Concomitantly, a ventral region of the follicular epithelium is delimited which will provide

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spatial cues governing the establishment of the embryonic dorsoventral axis.

grk encodes a protein of the TGF-α class of growth factors (Neumann-Silberberg and Schüpbach, 1993) that signals to the follicular epithelium through its receptor Torpedo/DER, the *Drosophila* homologue of the vertebrate EGF receptors (Price et al., 1989; Schejter and Shilo, 1989). Activation of the DER receptor tyrosine kinase by Gurken leads to the activation of the canonical Ras signal transduction pathway including Ras, Raf/*l(1)pole hole* MEK/*Dsor* and MAPK/*rolled* (for review see Ray and Schüpbach, 1996). However, within the dorsal follicular epithelium at least two different cell types are induced in response to the same intracellular cascade, namely the two respiratory appendages at dorsolateral positions and the cells separating them at the dorsalmost levels.

This opens up the question of how the local Gurken signal is received and processed in the follicular epithelium to generate long-range dorsoventral patterning. The problem is posed in an even more striking way by a comparison with *Drosophila virilis*. We demonstrate that in this species Gurken localization to the anterior dorsal corner of the oocyte occurs in exactly the same way as in *D. melanogaster*. Nevertheless, in response to this identical initial dorsalizing cue, four instead of two dorsal appendages are specified at different dorsoventral positions.

Two conflicting models have been proposed in the literature to explain this apparent discrepancy between local cause and global effect. On the one hand, much of the dorsoventral information could already be encoded by the Gurken protein distribution. Gurken could act as a graded morphogen, its local concentration determining spatial fates along the dorsoventral axis. Such models were proposed by Neumann-Silberberg and Schüpbach (1994), based on the dosage sensitivity of *grk,* and later by Deng and Bownes (1997), based on the dependency of *broad-complex* expression in the dorsal appendage anlagen on Gurken signaling intensity.

Alternatively, Sapir et al. (1998) provided evidence for the involvement of Spitz, another DER ligand, in a second wave of DER activation associated with patterning and refinement processes within the follicular epithelium. Under such a scenario much of the spatial information would not be provided by the Gurken signal but rather be generated by interactions within the follicular epithelium.

In this work we present experiments designed to distinguish between these two competing hypotheses. We show that Gurken protein is released from the oocyte and internalized by the follicle cells during signaling. We use this observation to demonstrate that the range of Gurken diffusion after secretion from the oocyte is very limited. In contrast to this static Gurken signal antibody staining against activated MAPK reveals a spatially and temporally changing activation pattern of the Ras pathway downstream of the Gurker receptor Top/DER.

We, therefore, carefully reevaluated the expression dynamics of Gurken target genes potentially able to exert positive and negative feedback on the DER pathway. We demonstrate that indeed MAPK activation is correlated with the expression of genes like *rhomboid* (Bier et al., 1990: Ruohola-Baker et al., 1993), *argos* and *sprouty*. Rhomboid has been shown to activate the agonistic DER ligand Spitz in other tissues (Schweitzer et al., 1995b: Golembo et al., 1996b). In addition, it has been suggested that Spitz is involved in DER activation during patterning of the follicular epithelium (Sapir et al., 1998). In contrast, *aos* (Schweitzer et al., 1995a) and *sty* (Hacohen et al., 1998) encode secreted inhibitors of DER and FGF receptor tyrosine kinase pathways, respectively. Analysis of the expression patterns in backgrounds with altered Gurken signaling activity, further supports the link between MAPK activation and the expression of potential modulators of DER activity and provides evidence that Gurken signaling continues to be integrated with positive and negative feedback loops determining the activation state of the DER pathway in the follicle cells.

To understand how MAPK activation is then translated into dorsoventral follicle cell fates we reanalyzed its relation to the expression of *broad-complex*, a gene previously described as a determinant of the dorsal appendage anlagen. Indeed MAPK activation levels presage the specification of different cell types along the dorsoventral axis.

In conclusion we suggest a new model of how local Gurken signaling by the oocyte is translated into long range dorsoventral patterning of the egg shell.

2. Results

2.1. grk mRNA is equivalently localized in oocytes from D. virilis and D. melanogaster despite the differences in egg shell morphology

To determine how tightly Gurken distribution and the patterning of the dorsal follicular epithelium are linked we examined *grk* RNA localization in oocytes from *Drosophila virilis*.

D. virilis is evolutionarily separated from *D. melanogaster* by about 60 Myr (reviewed in Ashburner, 1989) and despite the general similarity of oogenesis in the two species its egg shells bear four rather than two dorsal appendages (Fig. 1c,d) We cloned the Gurken homologue from *D. virilis* and could show that the two proteins possess a conserved domain structure. However, continuous stretches of high similarity at the sequence level are limited to a few short domains including the signal peptide and the transmembrane and intracellular domains. The overall identity of both proteins is 40.3% and their similarity including equivalent substitutions 45.7% (Fig. 2).

It was possible to identify equivalent stages of oogenesis in the two species by criteria not affected by the more elongated shape of the *D. virilis* egg chambers, such as the

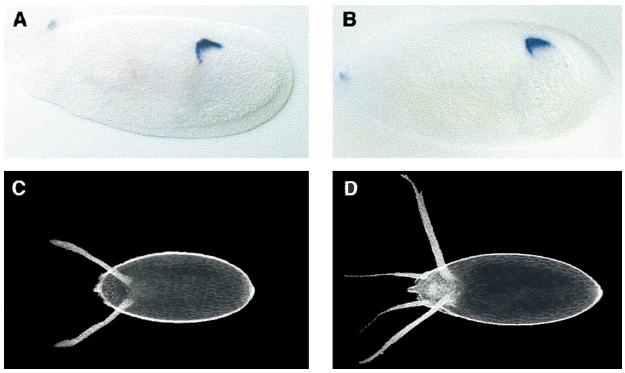

Fig. 1. Gurken mRNA is similarly localized in *Drosophila melanogaster* and *Drosophila virilis* oocytes. (A,B) In situ hybridizations on stage 9 egg chambers of (A) *D. melanogaster* and (B) *D. virilis* using species specific *grk*-RNA probes. The mRNAs are sharply localized to the dorsal anterior corner of the egg chambers in both species. (C,D) Dark field micrographs, dorsal surface views, anterior to the left. (C) Wild type egg of *D. melanogaster* displaying two respiratory appendages on its dorsal side, an operculum with a centrally located micropyle at its anterior tip, and an aeropyle at the rounded posterior pole. (D) The egg of *D. virilis* has four respiratory appendages on its dorsal side. One pair is more anteriorly located and it is close to the operculum. Micropyle and aeropyle are also visible.

beginning of yolk uptake, the migration of the oocyte nucleus, and the transition from uniform to stretched and columnar follicular epithelium. In situ hybridization revealed that throughout oogenesis *grk* RNA localization in *D. virilis* ovaries is highly similar to *D. melanogaster.* In both species *grk* RNA remains largely associated with the oocyte nucleus during all stages. Specifically, *grk* mRNA is also tightly localized to the dorsal anterior corner of the *D. virilis* oocytes at stages 8 to 10 of oogenesis (Fig. 1a,b). The only detectable difference was the retention of a significant amount of *grk* RNA at the posterior pole of *D. virilis* oocytes when the bulk of the RNA was already found at the dorsal anterior corner of the oocyte. This is actually also observable with *D. melanogaster gurken* RNA and protein, albeit at a quantitatively much lower level (data not shown).

Although the egg shells are patterned in a distinctly different way, in the oocytes of both species *gurken* RNA distribution limits the source of the dorsalizing signal to a very restricted region (Fig. 1a,b). This highlights the problem of how the very local initial dorsal cues can be translated into overall dorsoventral patterning of the follicle cells.

2.2. Gurken protein is released from the oocyte and internalized by the follicular epithelium in a locally restricted way

We have generated an antiserum against a *N*-terminal fragment of Gurken that allows us to detect the secretion of the protein from the oocyte and its uptake into follicle cells in association with both posterior and dorsal signaling events. This allows us to estimate how far the Gurken signal reaches once the protein is released from the oocyte.

Gurken staining in the follicular epithelium is detectable in small dotlike structures within individual follicle cells, presumably corresponding to vesicular compartments such as endosomes or lysosomes (see e.g. Hamel et al., 1997). Gurken internalization can first be observed in all posterior follicle cells abutting the oocyte from stage 4 to 6 of oogenesis (Fig. 3a), although *grk* RNA and protein accumulation in the oocyte can be detected at even earlier stages down to region 2A of the germarium (Neumann-Silberberg and Schüpbach, 1993; Neumann-Silberberg and Schüpbach, 1996). In wholemount stainings Gurken uptake is visible along the dorsal side of the follicular epithelium during stages 7 and 8 of oogenesis (Fig. 3b) and is clearly detectable only in a limited subset of follicle cells directly overlying the oocyte nucleus during stages 9 and 10 (Fig. 3c). Sections through stage 10 oocytes confirm that the uptake is restricted to a relatively narrow domain that does not extend laterally beyond the region of protein accumulation within the oocyte (Fig. 3d).

Analysis of Gurken pathway mutants demonstrated that the internalization of the Gurken protein into the follicular epithelium is associated with Gurken signaling. In dorsalized follicles from homozygous $K10^{13}$ females, Gurken protein is mislocalized and active around the anterior margin of the oocyte (Serano et al., 1995 Neumann-Silberberg and Schüpbach, 1996). Accordingly, in these ovaries Gurken internalization into follicle cells is detectable dorsally and ventrally around the circumference of the egg chambers (Fig. 3e). In contrast, no Gurken uptake by the follicular epithelium can be seen in the ventralized ovaries from females homozygous for the amorphic allele grk^{DC} despite the large amounts of protein present within the oocyte (Fig. 3f).

Gurken signaling can be directly observed only in a narrow domain of the follicular epithelium overlying the Gurken source within the oocyte. This domain does not significantly change its spatial extent during stages 9 and 10 of oogenesis. In addition, the detected level of Gurken uptake remains approximately constant and offers no indication of dramatic changes in signaling intensity throughout these stages.

2.3. MAPK shows a dynamic activation pattern in the follicular epithelium of wildtype egg chambers that is distinctly different from Gurken signaling

We next tried to determine how this spatial and temporal pattern of Gurken signaling is linked to the activation of Torpedo/DER pathway in the follicular epithelium. DER acts on the canonical Ras signal transduction cascade in the follicle cells. To directly visualize the activation state of this pathway we detected MAPK activation in the follicular epithelium using a polyclonal serum specifically recognizing a conserved phosphoepitope present only in

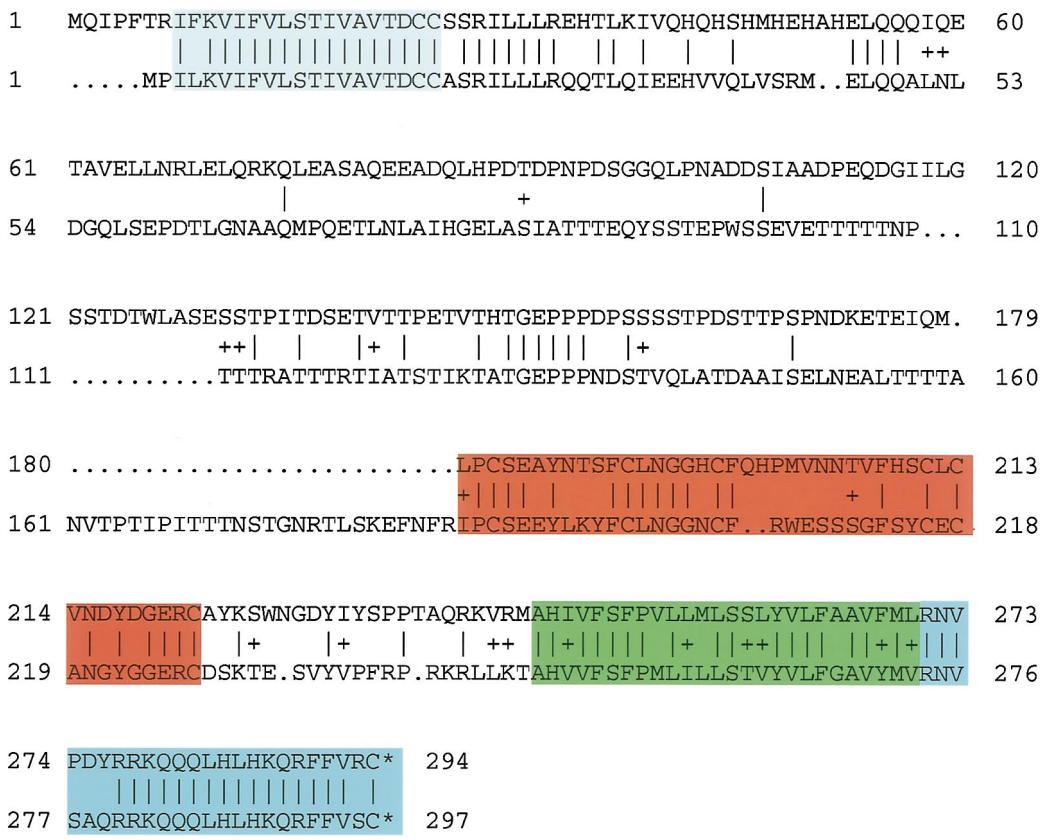

Fig. 2. Alignment of Gurken proteins from *Drosophila melanogaster* (top) and *Drosophila virilis* (bottom). The overall sequence identity is 40.3%, the similarity 45,7%. Continuous stretches of sequence conservation are confined to the signal peptide (light blue), the EGF repeat (orange), the transmembrane (green) and the cytoplasmic (dark blue) domains.

the activated form of the kinase (Gabay et al., 1997). In wild type *D. melanogaster* egg chambers, MAPK activation can first be detected in a single small group of about 30 cells located in the dorsalmost region of the epithelium abutting the oocyte nucleus at stage 10 of oogenesis (Fig. 4e,g). No MAPK activation is visible in younger stages. The small region of uniform activation then grows until it includes around 50–55 cells. The level of activation, as reflected by the intensity of the staining, starts to become stronger in the leading cells located at the periphery of the expanding group (Fig. 4h). The domain of MAPK activation then expands until it almost spans the dorsal half of the egg chamber at stage 10b (Fig. 4f). At the same time, the MAPK staining becomes weaker in two dorsolateral patches, but remains high along the dorsal mid-line and additionally becomes strongly activated in two lateral crescents. This results in a distinct 'spectacle' shape of staining with peripherally located cells showing high levels of activated MAPK surrounding two regions of more weakly stained cells which lie symmetrically at either side of the dorsal mid-line of the follicle (Fig. 4i). Later, at stage 11 of oogenesis MAPK is again strongly activated in the migrating cells forming the dorsal appendages (data not shown).

The dynamic spatial and temporal changes in the MAPK activation pattern during stage 10 of oogenesis occur at a time when, as shown above, the overall distribution of Gurken protein in the oocyte is static and the signal-associated uptake of Gurken remains limited to the dorsalmost follicle cells. In addition, MAPK activation only becomes detectable at stage 10 of oogenesis despite a clear requirement for prior MAPK function (Gonzalez-Reyes et al., 1995). As Gurken signaling at the same time does not appear to experience major changes in spatial extent and intensity, these observations suggest the presence of secondary amplification steps that raise the MAPK activation level over the detection threshold and may be responsible for the complex evolution of the activation pattern.

2.4. MAPK activation is closely linked to rhomboid expression in the follicular epithelium

The above results together indicate that MAPK activation does not simply reflect the input of Gurken on the Ras pathway downstream of Top/DER. We, therefore, looked for additional factors that could explain the observed complexity of the pattern. One gene with properties making it a good candidate for such a factor is *rhomboid* (Bier et al., 1990). *rho* expression is required and sufficient for the induction of

dorsal fates in the follicular epithelium (Ruohola-Baker et al., 1993). In the embryo, rhomboid protein can locally regulate the conversion of Spitz from an inactive ubiquitous membrane-associated precursor to an active diffusable growth factor (Schweitzer et al., 1995b; Golembo et al., 1996a), and may fulfil the same function in the follicular epithelium (Sapir et al., 1998). In addition, the dorsal expression of *rho* refines during stage 10 of oogenesis and is absent in strongly ventralized ovaries from *grk* females (Ruohola-Baker et al., 1993)

We, therefore, analyzed the correlation between the spatial and temporal dynamics of MAPK activation and the *rho* expression pattern. In wildtype egg chambers *rho* can first be detected in a broad domain centered on the anterodorsal corner of the oocyte at the transition from stage 9 to stage 10a of oogenesis (Ruohola-Baker et al., 1993), at a time when MAPK activation cannot yet be detected in the follicular epithelium. Thereafter, *rho* RNA starts to be downregulated dorsally, beginning at the anterior margin of the domain (Fig. 4a). Downregulation proceeds until the remnants of the first broad expression domain are reduced to a wide ring. *rho* expression is, at that time, retained only in a small patch in the dorsalmost cells and in two stripes extending laterally towards the nurse cell border. In these cells *rho* expression is then strongly upregulated (Fig. 4b) and a refinement process begins that leads to a final pattern consisting of two L-shaped domains (Fig. 4 c,d; Ruohola-Baker et al., 1993).

Just as *rho* refinement starts in the dorsalmost part of the egg chamber (Fig. 4b), MAPK activation in the follicular epithelium first exceeds the detection threshold in exactly the same cells (Fig. 4e,g). During the following expansion of the MAPK domain the strong activation at the leading edge (Fig. 4h) seems to coincide with the refining stripes of *rho* expression (Fig. 4c). As the MAPK activation pattern reaches its final spectacle shape (Fig. 4i), again the dorsal and anterior portions of the MAPK staining pattern are associated with *rho* expression (Fig. 4d).

In conclusion we demonstrate that *rho* expression precedes the detection of MAPK activation and that both stain-

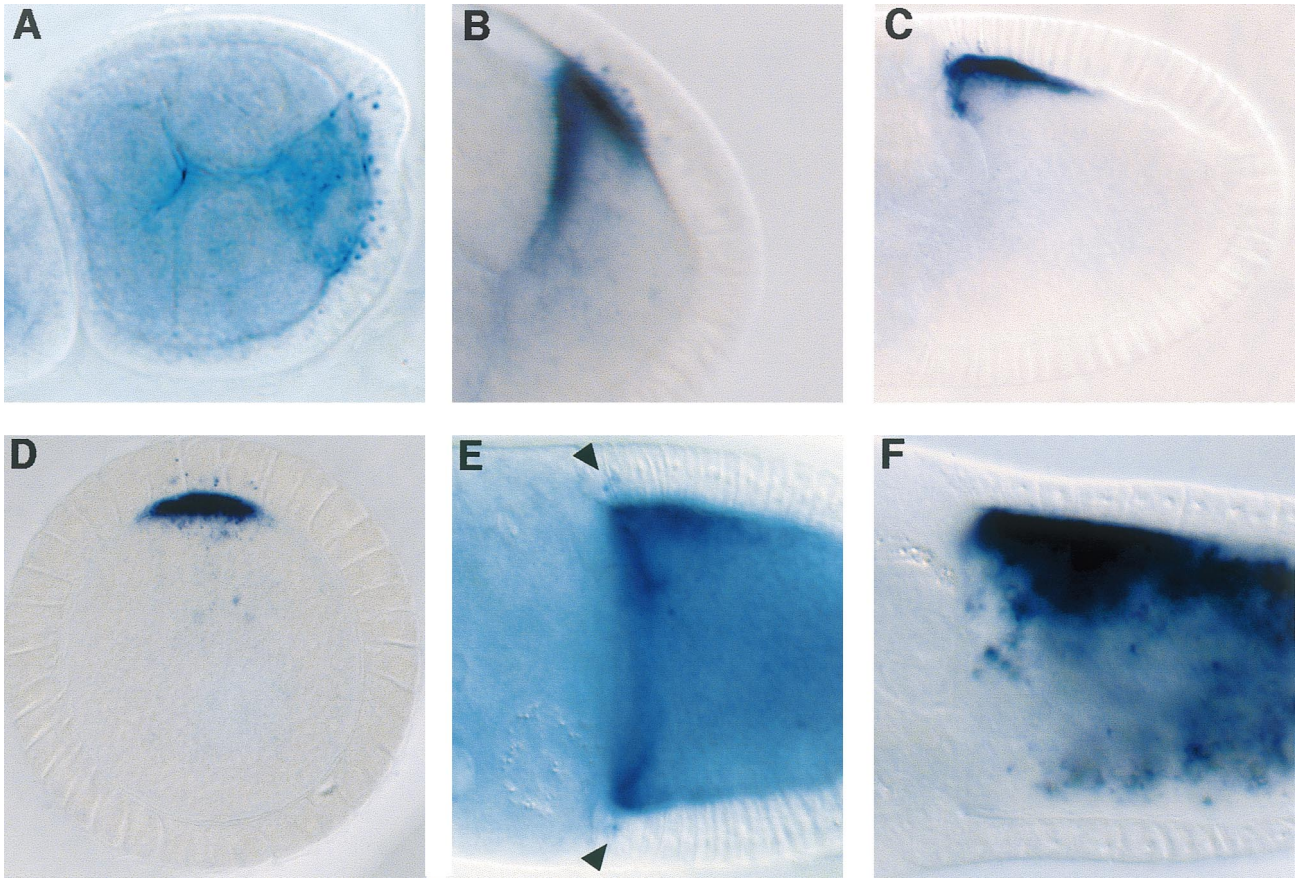

Fig. 3. During signaling Gurken is released from the oocyte and internalized by the follicular epithelium. (A–F) Egg chambers stained with anti-Gurken antiserum. (A–C) In the wild type egg chambers the uptake of Gurken protein in response to the Gurken signal is visible in all posterior follicle cells contacting the oocyte at (A) stage 4 and in dorsal follicle cells adjacent to the oocyte nucleus at (B) stage 8 and (C) stage 10A. (D) Cross-section of a wild type egg chamber at stage 10. Gurken internalization is restricted to the follicle cells overlying the region of Gurken accumulation within the oocyte. (E) $K10^{13}$ egg chamber. Gurken protein is mislocalized and active around the whole anterior margin of the oocyte. Gurken internalization by follicle cells is detectable dorsally and ventrally around the circumference of the egg chamber. (F) grk^{DC} egg chamber. A large amount of inactive protein is present inside the oocyte. In contrast to wild type, no Gurken uptake by the follicular epithelium can be detected.

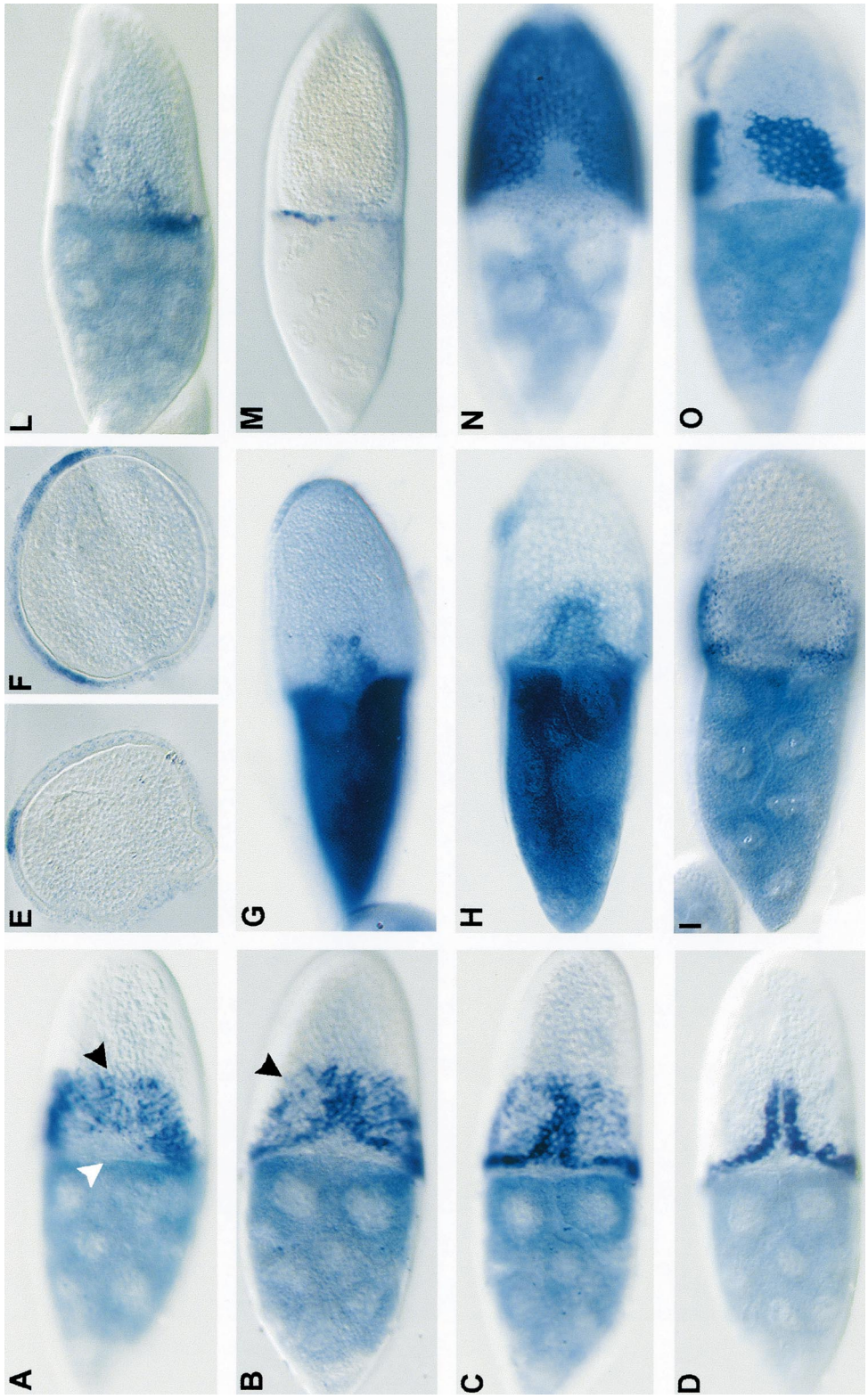

Fig. 4. *broad-complex* expression is correlated with dynamic *rhomboid* expression and MAPK activation in the follicular epithelium. (A–D) *rho* in-situ hybridizations on wildtype ovaries. (E–I) Stainings of wild type egg chambers with an antibody against activated MAPK. (L,M) *rho* RNA in weakly ventralized cnl^{CF5}/cnl^{AR55} egg chambers. (N,O) *broad-complex* expression in stage 10 wildtype follicles. (A–D) Refinement of the wild type *rho* expression pattern. (A) The initial broad and uniform *rho* expression (black arrowhead) is downregulated in an anterior domain starting near the oocyte nucleus (white arrowhead). (B) *rho* expression is upregulated in a narrow dorsal region and in two one cell-wide stripes extending form this dorsal domain towards the nurse cell border. During this stage the remnants of the early expression are still visible (black arrowhead). (C) The dorsal *rho* expression finally refines into two narrow stripes. (D) While the two dorsal *rho* stripes extend posteriorly, the remnants of the early broad domain vanish completely. (E–I) Dynamic activation pattern of MAPK in wild type. (E,G) MAPK activation can first be detected in a single small group of cells located in the dorsalmost region of the epithelium. (H) Around stage 10A activated MAPK can be detected in a larger domain. The level of activation is higher in the cells located at the periphery. (F) At stage 10B the domain of MAPK activation expands until it snaps the dorsal half of the egg chamber. The section is close to the oocyte-nurse cell border. (I) The activation becomes weaker in two dorsolateral patches and evolves into a distinct 'spectacle' shaped pattern. Despite the similarity this represents a later phase than the transient *rho* expression patterns in 4B and C. (L,M) In cnl^{AR55}/cnl^{CF5} egg chambers *rho* expression is present at stage 10A (L) but then disappears rapidly (M). No upregulation or refinement of the *rho* expression is detectable. (N,O) *BR-C* expression is associated with low levels of MAPK activation during appendage specification. (N) Repression of *BR-C* coincides with (H) high levels of MAPK activation in the dorsalmost follicle cells. (O) Late *BR-C* expression becomes confined to (I) the dorsolateral domains of low MAPK activation.

ing patterns are spatially and temporally well correlated. Rhomboid thus may be one of the factors responsible in generating the amplification and modulation of the initial Gurken signal.

2.5. Loss of MAPK activation and rho expression are correlated in hypomorphic gurken and cornichon mutants

We next analyzed the influence of mutations affecting Gurken signal strength on the link between MAPK activation and rhomboid expression in the follicular epithelium. As earlier described by Ruohola-Baker et al. (1993), severely reducing Gurken signaling will abolish *rho* expression in the dorsal follicular epithelium. In the weakly ventralized egg chambers from females carrying the hypomorphic allele combinations cni^{CF5}/cni^{AR55} or grk^{WG12}/grk^{HK36} dorsal fates are still clearly specified. However, eggs laid by such females have lost their dorsalmost fates and possess only one medially fused dorsal appendage. In these egg chambers MAPK activation in the follicular epithelium during stage 10 always remains below the detection threshold. This correlates well with the observed pattern of *rho* expression. While the early broad *rho* domain is specified correctly at stage 10a of oogenesis (Fig. 4l), it will then rapidly decay and upregulation and refinement do not occur (Fig. 4m).

We, therefore, conclude that *rho* expression and MAPK activation in the follicular epithelium are not self sustaining once initiated by Gurken. Instead, Gurken signaling exceeding a certain threshold continues to be required for the maintenance and refinement of the *rho* expression pattern.

2.6. Gurken signaling also induces the expression of negative elements in the DER pathway

Why does Gurken-induced expression of *rho* not lead to a runaway positive feedback loop dorsalizing the entire follicular epithelium? Clearly, some form of negative feedback has to limit this process as in principle even the ventralmost follicle cells are capable of adopting dorsal fates (e.g. in *K10* mutant ovaries). So far only one inhibitor of DER signaling has been shown to be induced in the dorsal follicular epithelium in response to DER activation.

The *argos (aos)* gene encodes a diffusible inactivating DER ligand (Schweitzer et al., 1995a) and is expressed in a small transverse line across the dorsal and anteriormost follicle cells overlying the oocyte at stage 10b (Fig. 5a). During stages 11 and 12 *aos* expression expands into a narrow T-shaped domain at the dorsal anterior corner of the egg chamber (Fig. 5b; Queenan et al., 1997). However, this expression comes too late to explain the dynamic evolution of the MAPK and *rho* patterns during stage 10 of oogenesis.

In contrast, *sprouty (sty)* (Hacohen et al., 1998) is expressed early enough to be a part of the regulatory network controlling the DER pathway activation at stage 10. Just as *aos, sty* also encodes a cysteine-rich secreted factor. Sprouty has so far been shown to downregulate signaling by the *Drosophila* FGF receptor homologue Breathless during tracheal morphogenesis. Nevertheless the authors of this study suggested that Sprouty may also act as an inhibitor of other receptor tyrosine kinase signaling pathways (Hacohen et al., 1998). We show that *sty* expression is induced in all posterior follicle cells abutting the oocyte at stages 4 to 6 of oogenesis. The dorsalizing Gurken signal also causes an immediate *sty* response in the dorsal follicular epithelium during stages 7 to 9 (Fig. 5c). By stage 10, *sty* is expressed in a domain of intermediate width overlying the oocyte nucleus (Fig. 5d). In contrast, *sty* expression is absent from corresponding egg chambers lacking Gurken function (data not shown). Thus, *sty* may be one factor required to counteract the potential *rho*-dependent autoactivation of the DER pathway.

2.7. High levels of MAPK activation and expression of broad-complex occur in complementary domains during the specification of the dorsal appendage anlagen

To correlate DER pathway activation with the adoption of a specific dorsoventral fate we analyzed the relation between MAPK activation and *broad-complex* expression in the follicular epithelium.

The gene *broad-complex (BR-C)* encodes a family of alternatively spliced zinc-finger transcription factors (DiBello et al., 1991; Bayer et al., 1996). One of the *BR-C* isoforms has already been shown to be expressed in the follicle cells giving rise to the dorsal appendages. This *BR-C* function is required for appendage formation and is sensitive to mutations in the Gurken signal transduction pathway (Deng and Bownes, 1997), leading to the proposal that *BR-C* actually is the determinant of the dorsal appendage anlagen. However, analysis of the *BR-C* pattern is complicated by the fact that it seems to be generally expressed in cells that will undergo morphogenetic movements.

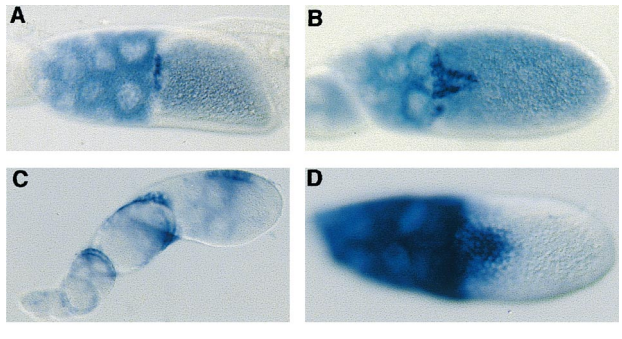

Fig. 5. *argos* and *sprouty* are expressed in the follicular epithelium. (A,B) *aos* in situ hybridizations on wild type ovaries. (A) *aos* expression starts at stage 10A in an anterior transverse stripe which then (B) evolves into a T-shaped domain that splits around stage 11. (C,D) *sty* in situ hybridizations on wild type ovaries. (C) *sty* is expressed in all posterior follicle cells in contact with the oocyte at stage 4 of oogenesis. (D) During stage 9 and 10 *sty* is strongly expressed at the dorsal side of the egg chambers.

Support for this interpretation comes from our observations in embryos, where *BR-C is* specifically upregulated in most tissues undergoing major morphological changes. For example, at gastrulation higher expression levels can be detected in the regions where the ventral furrow, the head fold and the dorsal folds will form (Fig. 6a).

During oogenesis, *BR-C* is expressed in two clearly separable phases. In agreement with the observations of Deng and Bownes (1997) we can first detect *BR-C* expression uniformly in all cells of the follicular epithelium at stage 6 of oogenesis (Fig. 6b). During this phase, expression is independent from Gurken inputs as it is not altered in *grk* or *cni* mutant follicles (Fig. 7c; Deng and Bownes, 1997). During stages 7 through 9 of oogenesis the cells of the follicular epithelium stretch over the nurse compartment, migrate posteriorly and later form a dense columnar epithelium over the growing oocyte. At the beginning of stage 9 all follicle cells still ubiquitously express *BR-C*. Later in the same stage, *BR-C* expression begins to decay starting from both poles of the egg chamber, which again is well correlated with the places where stretched and columnar follicle cells first reach their final position and shape (Fig. 6b,c).

Around the transition from stage 9 to 10 of oogenesis *BR-C* expression starts to become influenced by DER signaling (Deng and Bownes, 1997). From then on the *BR-C* expression pattern is highly correlated with MAPK activation levels. *BR-C* is rapidly downregulated in a sharply delineated patch above the anterodorsal corner of the oocyte during stage 10a (Figs. 4h and 6d). This domain coincides with the first strong peak of MAPK activation resulting in complementary staining patterns for *BR-C* and activated MAPK (Fig. 4h,n). As the regions of anterodorsal MAPK dependent repression and polar decay of the staining expand, *BR-C* expression becomes confined to the two dorsolateral patches identified by Deng and Bownes (1997) as appendage anlagen (Figs. 4o and 6d). These patches are coincident with the two central domains of weakly activated MAPK surrounded by regions of stronger MAPK activation described above (Fig. 4i,o).

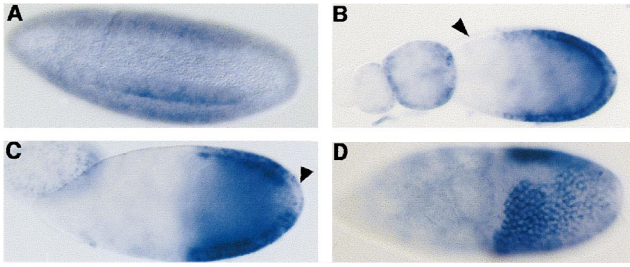

Fig. 6. Expression of *BR-C* is correlated with morphogenetic movements during embryogenesis and oogenesis. (A–D) In situ hybridizations using a *BR-C* specific RNA probe. (A) *BR-C* is expressed during gastrulation in the regions where the ventral furrow and the head and dorsal folds will form. (B) At stage 6 of oogenesis *BR-C* is detectable in all follicle cells. Expression begins to decay from the anterior pole at stage 9 (black arrowhead). (C) Around the transition to stage 10 *BR-C* expression continues to decay from both poles of the egg chamber (black arrowhead). (D) Later in stage 10, *BR-C* expression is rapidly downregulated dorsally. At the same time it continues to decay ventrally and posteriorly, gradually becoming confined to two lateral patches.

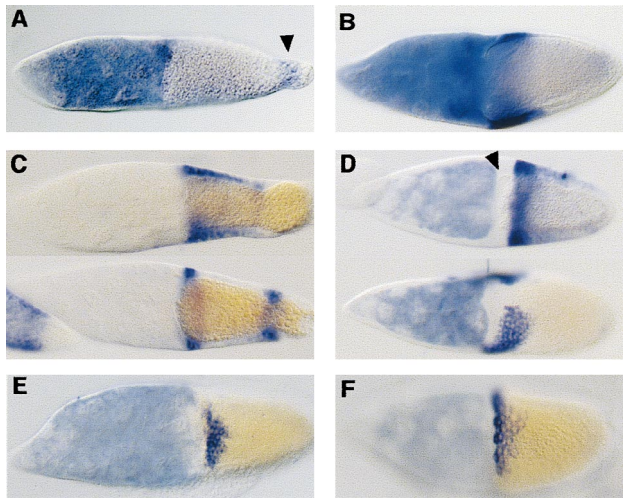

Fig. 7. MAPK activation and *BR-C* expression in mutant egg chambers. (A,C) $cni^{AR55}/Df(2L)H60$ egg chambers. In these egg chambers Gurker signaling is completely abolished. (A) Antibody stainings against activated MAPK and (C) in situ hybridizations using a specific-BR-C Probe. (A) MAPK is activated at the posterior of the follicular epithelium (arrowhead). (C). *BR-C* is first uniformly expressed in the follicular epithelium (upper egg chamber). Later the expression resolves into two rings around the duplicated anterior follicle cells (lower egg chamber). (B,D) $K10^{13}$ egg chambers. (B) Antibody stainings against activated MAPK and (D) in situ hybridization using a specific-*BR-C* probe. (B) MAPK activation is detected in all follicle cells around the anterior end of the oocyte at stage 10. (D) *BR-C* is expressed in a narrow ring abutting the posterior margin of the activated MAPK domain (upper egg chamber, the arrowhead marks the position of the oocyte nucleus). Later (lower egg chamber). the *BR-C* expression is lost in dorsal cells, but is maintained at the ventral side corresponding to the expanded dorsal appendage anlagen. (E–F) *BR-C* in situ hybridization on hypomorphic *cornichon* ovaries. In ventralized egg chambers from (E) the intermediate strength allele combination cni^{AR55}/cni^{AA12} and from (F) the weak hypomorphic combination cni^{AA55}/cni^{CF5} egg chambers *BR-C* expression is restricted to single patches of cells directly overlying the oocyte nuclei.

We conclude that the dorsal appendage anlagen are specified within regions of low MAPK activation during stage 10 of oogenesis. However, the same cells will later show high levels of MAPK activation as they migrate towards the dorsal anterior tip of the forming egg during morphogenesis of the appendages (data not shown). This later MAPK activation is presumably caused by the late *rho* expression described by Sapir et al. (1998).

2.8. Both BR-C expression and MAPK activation pattern are altered in cni and K10 ovaries

Elimination of the Gurken signal results in a complete loss of dorsal fates in the follicular epithelium. Nevertheless, we could detect a ring of ectopically activated MAPK at the posterior end of $cni^{AR55}/Df(2L)H60$ ovaries that exhibit no trace of Gurken activity (Fig. 7a). How-

ever, this activation occurs in follicle cells that have lost their columnar epithelial organization as a consequence of misspecification of the anteroposterior axis of the egg chamber and have assumed border cell and centripetal follicle cell fates (Gonzalez-Reyes et al., 1995; Roth et al., 1995). Therefore, the ectopic activation of MAPK cannot be related to a induction of dorsal follicle cell fates in these cells.

As previously described by Deng and Bownes (1997), the early *BR-C* expression is not affected in ventralized follicles. However, in the absence of Gurken signaling *BR-C* expression does not disappear in later stages of oogenesis. Rather, in stage 10 cni^{AR55}/*Df(2L)H60* follicles the pattern will resolve into two rings around the duplicated anterior follicle cells (Fig. 7c). As no trace of dorsolateral structures is visible in the ventralized egg shells, this observation rules out that *BR-C* expression is the determinant of appendage fate. Nevertheless, as Deng and Bownes (1997) have demonstrated, *BR-C* expression can serve as a useful marker for the dorsal appendage anlagen as long as this fate is specified.

In dorsalized egg chambers from females homozygous for the strong *K10* allele $K10^{13}$ Gurken protein is mislocalized and active around the anterior cortex of the oocyte at the relevant stages (Serano et al., 1995; Neumann-Silberberg and Schüpbach, 1996). As expected, we were able to detect MAPK activation in all follicle cells around the anterior end of the oocyte at stage 10 (Fig. 7b). In the mutant egg chambers the ubiquitous expression of *BR-C* refines to a narrow ring abutting the posterior margin of the domain of activated MAPK. This ring includes the dorsalmost cells giving rise to interappendage material and persists until late stage l0b of oogenesis. Finally, the expression in the dorsalmost cells is lost and *BR-C* expression becomes restricted to the ventrally expanded dorsal appendage anlagen, although this refinement occurs at a later stage than in wildtype follicles (Fig. 7d).

As mentioned above, MAPK activation always remains below the detection threshold in the follicular epithelium of egg chambers from females mutant for the hypomorphic allele combinations cni^{CF5}/cni^{AR55} and cni^{AA12}/cni^{AR55}. In these mutant ovaries *BR-C* is expressed in a single patch of cells directly overlying the oocyte nucleus, the high point of the residual Gurken signal (Fig. 7e,f). In correlation with the absence of detectable levels of MAPK activation, no dorsal splitting of this *BR-C* domain into lateral patches could be observed.

We conclude that *BR-C* expression is excluded from regions where MAPK activation exceeds the detection threshold due to DER signal amplification, as shown by the staining patterns in wildtype and *K10* follicles. However, analysis of the ventralized follicles demonstrates that a certain degree of DER pathway activation corresponding to a MAPK activation below the detection threshold is necessary to specify the appendage anlagen, which will in turn reactivate or retain *BR-C* expression.

2.9. MAPK activation evolves in a dynamically different way in Drosophila virilis ovaries

Dynamic evolution of the MAPK activation pattern could also be detected in *D. virilis*. In stage 10a follicles, MAPK activation is visible all along the dorsal mid-line (Fig. 8a). This domain will later refine into two dorsolateral patches excluding a triangular region overlying the domain of high Gurken concentration near the oocyte nucleus (Fig. 8b). These lateral patches will not refine further into four ringlike domains corresponding to the 'spectacle' pattern of MAPK activation in *D. melanogaster*. However, the two lower appendages are formed so far anteriorly that MAPK activation in or near their anlagen may likely be masked by the nurse cell staining. Nevertheless, we are not able to detect paired regions of weak MAPK activation that could presage specification of the appendage anlagen in the same way as in *D. melanogaster*.

We conclude that in both species the similar initial Gurken signal is amplified and modulated resulting in two different MAPK activation patterns. Furthermore, the resulting egg shells differ strikingly between the two species. Therefore, both the processes controlling the activation patterns of MAPK and the ways these patterns are interpreted to specify the appendage anlagen must have evolved differently in the two species.

3. Discussion

Dorsoventral patterning of the developing *Drosophila* egg is governed by the activation of the EGF-receptor homologue Top/DER through Gurken, a ligand emanating from a spatially restricted source in the oocyte. However,

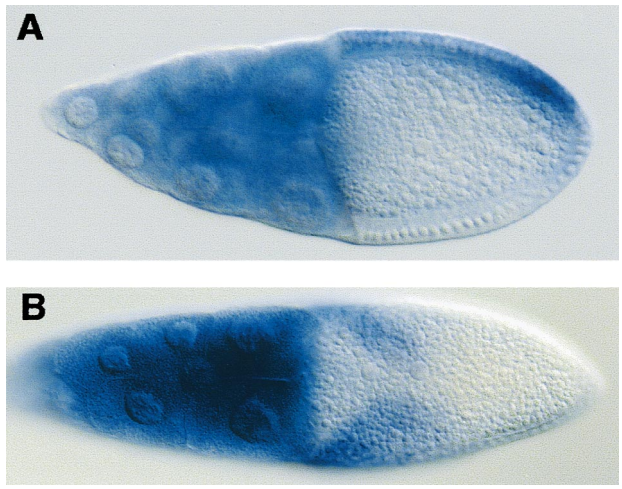

Fig. 8. MAPK activation pattern in *Drosophila virilis*. (A,B) Stainings of *D. virilis* egg chambers with an antibody against activated MAPK. (A) At stage 10 of oogenesis MAPK is activated in a dorsal stripe extending to the very posterior of the egg chamber. (B) Later, MAPK activation is excluded from the dorsalmost cells and is confined to two triangular lateral domains.

the consequences of this signal are not just local in nature. Rather, spatial fates in the entire follicular epithelium are affected. To explain this apparent discrepancy between cause and effect, two extreme models are conceivable.

Radically extending the proposal of Deng and Bownes (1997), the long range pattern could be a consequence of the precise readout of a morphogen gradient. A Gurken protein concentration gradient with a dorsal high point and declining levels towards the ventral side could, by activating or repressing target gene expression at different ligand concentration thresholds, specify distinct dorsoventral fates without subsequent refinement processes occurring in the follicular epithelium. Alternatively and equally extreme, Gurken signaling could merely initiate and position a secondary signaling network patterning the follicular epithelium without relying on further input from the oocyte. Without claiming a exclusive role for such a process, the existence of a second round of DER activation in dorsoventral patterning of the follicular epithelium has previously been suggested by Sapir et al. (1998). Our results favor an intermediate model that incorporates aspects of both these proposals.

Gurken signaling stimulates, sequentially, two subsets of cells within the follicular epithelium to adopt posterior and dorsal fates, respectively (Gonzalez-Reyes et al., 1995; Roth et al., 1995). In association with both signaling events we were able to detect Gurken protein internalization into follicle cells responding to the signal, demonstrating that Gurken protein is indeed released from the oocyte. By analyzing inactive and ectopically signaling mutants we could show that uptake into the follicular epithelium occurs specifically in association with Gurken signaling. Most likely, this internalization is involved in the downregulation of the Gurken signal. It has been shown that other members of the TGF-α class of signal molecules that occupied active receptors can be cleared from the target membrane together with their bound ligand. The ligand is then proteolytically degraded in lysosomes while the free and inactive receptor may be recycled to the plasma membrane (e.g. see Dempsey and Coffey, 1994). This interpretation also could explain the loss of Top/DER receptors from the membrane of dorsal follicle cells responding to Gurken described by Sapir et al. (1998), although the authors of this study blame at least part of the decrease on transcriptional downregulation.

We could demonstrate that Gurken uptake is limited to the cells directly overlying the region of high Gurken concentration inside stage 10 oocytes. Uptake is also detectable in all posterior follicle cells in contact with the oocyte at stage 4 to 6. The number of these cells, and therefore, the number of cells internalizing Gurken at this stage, is equivalent to the number of cells that can be forced to adopt anterior fates by removing DER function (Gonzalez-Reyes and St. Johnston, 1998). Hence, during the first round of signaling we can detect internalized protein in all cells responding to Gurken under normal conditions. The number of cells that are stimulated by Gurken but internalize the protein at levels too low for detection is, therefore, likely to be very small, and the range of Gurken diffusion is minimal. Diffusion of Gurken between oocyte and follicular epithelium will be even more impeded by the beginning of the deposition of extraembryonic membrane material and the rough surface morphology of both plasma membranes at stages 9 and 10, when the membrane surfaces are covered by microvillous processes to facilitate rapid yolk uptake (King, 1970). The observation that activation of several target genes like *kekkon* (Musacchio and Perrimon, 1996) or *sprouty* (Fig. 5c) is strictly limited to the cells directly in contact with the oocyte during posterior signaling, supports the idea that lateral diffusion of Gurken protein after shedding is minimal. Therefore, a Gurken protein gradient generated by diffusion from a local source cannot be invoked to explain long range patterning of the follicular epithelium.

Using protein internalization in the follicular epithelium as a marker we could demonstrate that Gurken signaling occurs in a static fashion during stage 10b of oogenesis. In contrast, at the same stage MAPK is activated in a dynamically evolving spatial and temporal pattern. MAPK is a downstream element of the canonical Ras pathway known to be active downstream of the putative Gurken receptor Top/DER (for review, see Ray and Schüpbach, 1996). Interestingly, MAPK activation starts to be detectable much later than Gurken uptake in the same dorsal target cells. This difference cannot be explained by a requirement for a longer phase of signal accumulation. As shown in mammalian cells, the time scales for MAPK activation in response to TGF-α signaling molecules normally are in the order of seconds rather than minutes (e.g. see Schwenger et al., 1996). In addition, signal transduction and clearance of the receptor, that in this case can already be observed at younger stages than MAPK activation, typically occur almost coincidentally (Dempsey and Coffey, 1994; Bergeron et al., 1995). Therefore, signal amplification and modulation by additional inputs into the DER pathway have to be invoked in order to explain the different temporal and spatial patterns of Gurken signaling and MAPK activation.

This idea is reinforced by a comparison with *D. virilis*, a species separated from *D. melanogaster* by about 60 Myr of evolution. Here, the initial dorsalizing cue, namely *grk* RNA localization, occurs in exactly the same way as in *D. melanogaster.* Nevertheless, the final pattern of the egg shells is strongly divergent and already the mechanisms controlling the spatio-temporal dynamics of MAPK pathway activation have evolved in distinctly different ways.

We therefore, reanalyzed the expression of known Gurken target genes to correlate their expression with MAPK activation. Several candidates are described that could be capable of exerting positive or negative feedback on the DER pathway. *rhomboid* encodes a seven pass transmembrane protein required for the activation of Spitz, another TGF-α family DER ligand (Schweitzer et al., 1995b; Golembo et al., 1996a). It had been shown previously that *rho* is required for the correct dorsoventral patterning of the

follicular epithelium and is a target gene of DER signaling (Ruohola-Baker et al., 1993). During stage 10a of oogenesis *rho* is first expressed in a broad dorsal domain in the follicle cells overlying the anterior end of the oocyte. The expression then becomes weaker within this domain and strong dorsal *rho* expression starts to be detectable above the oocyte nucleus around the beginning of stage 10b. The *rho* domain will then refine into two stripes extending towards the posterior of the egg chamber. *rho* upregulation and refinement coincide with the first visible activation of MAPK in the follicular epithelium. The small initial MAPK activation domain will then expand, its more intensely stained leading edge following the expression of *rho*. This suggests a positive feedback of *rho* on its own activating pathway. Finally MAPK will additionally become activated in two lateral crescents resulting in a distinct 'spectacle shaped' staining pattern.

The observation that the positive feedback between *rho* and DER activation does not result in runaway activation of the pathway in the entire follicular epithelium suggests the presence of counteracting negative elements in DER dependent follicle cell patterning. The expression of one inhibitory DER ligand, namely Argos, in the follicular epithelium has already been described (Queenan et al., 1997). However, strong dorsal *aos* expression appears to start only after the dynamically evolving phase of MAPK activation. We suggest the Sprouty, another molecule presumably capable of inhibiting DER function, is expressed in a Gurken dependent manner and early enough to potentially exert negative feedback on DER activation following *rho* expression.

Are these secondary amplification and refinement processes acting on the DER pathway during stage 10 independent from further inputs from Gurken, once they are initiated in the follicular epithelium? We do not think so. Rather, Gurken signaling seems to be continuously required as one of several signals integrated to determine DER activation in the follicular epithelium. The reduced Gurken function in weak *grk* or *cni* hypomorphic backgrounds that clearly possess some dorsal fates is still sufficient to initiate the broad early *rho* expression. However, subsequently the dorsal expression will vanish completely, presumably due to the action of DER-dependent repressors in the follicular epithelium. In these mutant ovaries *rho* upregulation and refinement do not occur. Consequently, no activated MAPK can be observed during stage 10b, arguing that a positive feedback by *rho* on DER activation is crucial for the amplification required to raise MAPK activation levels over the detection threshold.

Furthermore, we suggest that the strong dorsal upregulation of *rho* expression and the subsequent refinement into the two dorsal stripes is directly dependent on continued Gurken signaling from the oocyte. We propose that in weak *cni* or *grk* mutants the strength of the Gurken signal is reduced below a critical threshold, below which the total activating inputs on the DER pathway are no longer able to counteract the inhibitory feedback, generated in the follicular epithelium.

In this context it is worth noting that Gurken, emanating from the oocyte, is totally insensitive to negative feedback through DER-mediated transcriptional downregulation. This distinguishes the Gurken signal from any positive feedback on the DER pathway by ligands produced in the follicular epithelium. A continued local requirement for Gurken, in contrast to a pure initiation function, could therefore, serve to stably anchor the secondary patterning processes, specify the dorsalmost fates and also explain the dosage sensitivity of this gene (Neumann-Silberberg and Schüpbach, 1994). A sensitive dependence of the upregulation and maintenance of *rho* expression on continued Gurken signaling could also explain the inability of ectopically supplied *rho* to dorsalize *grk* mutant egg chambers (Ruohola-Baker et al., 1993) We suggest that a transient pulse of *rho* overexpression may fail to support further *rho* production due to an overwhelming influence of DER-dependent inhibitors that *rho* itself had activated. In contrast, under such a model continued supply of *rho* may well dorsalize the follicular epithelium, as it has already been demonstrated by Sapir et al. (1998).

In the absence of Gurken signal, as e.g. in *cni* null mutations, MAPK is ectopically activated at the posterior end of the follicular epithelium. MAPK activation is detectable in cells that have lost their columnar epithelial organization by stage 10 of oogenesis. This probably reflects activation of the pathway, not downstream of the DER but rather via the *Drosophila* FGF receptor Breathless, that has been shown to be active in the migrating border cells (Murphy et al., 1995). MAPK activation in moving border cells from wildtype follicles would be masked by the strong staining in the surrounding nurse cells.

How does MAPK activation influence the morphogenesis of the developing egg shell? Deng and Bownes (1997) demonstrated that the gene *BR-C* is expressed and required in the anlagen of the dorsal respiratory appendages. They suggested, therefore, that *BR-C* acts as a marker specifying appendage fate and proposed a morphogen gradient model for Gurken signaling based on the sensitive dependence of *BR-C* expression on Gurken signal strength. Indeed, Gurken signaling leads to a repression of *BR-C* in the dorsalmost cells of the follicular epithelium. *BR-C* expression disappears in a sharply delineated dorsal–anterior patch coincident with the first observable MAPK activation. Later when the MAPK activation refines to its distinct 'spectacle shape', *BR-C* expression is confined to two lateral domains showing weak MAPK activation surrounded by rings strongly staining for activated MAPK. Based on the striking complementarity of these patterns we propose that at this stage *BR-C* is repressed by high levels and activated by low levels of MAPK activation.

In hypomorphic mutations reducing DER pathway activation in the follicular epithelium the appendage anlagen is shifted dorsally towards the highpoint of the residual Gur-

ken signal. However, we do not believe that this reflects the readout of a shallower Gurken morphogen gradient. Instead, for the reasons stated above, it appears that the residual Gurken signal is insufficient to initiate *rho* upregulation and refinement. The resulting absence of detectable levels of MAPK activation in the dorsalmost follicle cells then permits specification of appendage fates. However, since MAPK activation levels do not directly reflect local Gurken concentration, the observed shifts in *BR-C* expression following manipulation of Gurken signaling intensity cannot serve as evidence for a Gurken morphogen gradient.

In addition, *BR-C* expression does not by itself specify dorsal appendage fate, as it is visible in duplicated anterior ringlike domains in completely ventralized ovaries that do not posses appendages. This adds a caveat to using *BR-C* expression as a fate marker for dorsoventral positions. We propose that BR-C instead is general marker expressed in cells that undergo morphogenetic changes, consistent with its expression in the embryo and during earlier stages of oogenesis. Admittedly, in stage 10b follicles such a requirement is largely limited to the appendage anlagen as long as these are specified.

In conclusion we would, therefore, like to propose an intermediate model for the role of Gurken in the dorsoventral patterning of the follicular epithelium: Gurken does not act as graded morphogen and the dorsalizing Gurken signal does not by itself contain all the spatial information ready to be read out by the follicular epithelium and translated into distinct dorsoventral fates. Instead, it initiates secondary processes in the follicular epithelium that modulate and amplify the initial activation of the DER pathway. However, requirement for Gurken does not end with this. Rather, Gurken signaling continues to be integrated into the DER response and is needed at levels exceeding certain thresholds to locally overcome the influence of negative feedback loops on DER pathway activation. In this context it may be crucial that the Gurken signal remains strictly local and its production in the oocyte cannot be downregulated by DER activation, thereby distinguishing it from positive inputs on the DER pathway generated in the follicular epithelium.

4. Experimental procedures

4.1. Fly stocks

The following *D. melanogaster* strains were used: *Oregon* R, *fs(1)Kl0*^{l3} (Wieschaus et al., 1978), *cni*^{AR55} and *cni*^{AA12}, (Ashburner et al., 1990). *cni*^{CF5} was isolated in a screen for lethal and female sterile mutations uncovered by *Df(2L)H60-3* (Roth et al., 1995). grk^{WG}, grk^{HK} are described by Schüpbach (1987) and Neumann-Silberberg and Schüpbach (1993). All stocks were raised on standard corn meal food at room temperature (around 25°C).

4.2. RNA in situ hybridizations

The *broad-complex* core domain (Deng and Bownes, 1997), *rho* (Bier et al., 1990), *aos* (Freeman et al., 1992) and *sty* (Hacohen et al., 1998) transcripts were detected by in situ hybridization with digoxigenin labeled antisense RNA probes. The *sprouty* probe was raised against the EST HL04904 from the Berkeley *Drosophila* Genome Prolect. The *D. virilis gurken* probe was derived from a genomic fragment containing the coding region. The probes were labeled according to the protocol of the supplier of the labeling kit (Boehringer–Mannheim). The hybridization procedure was a modification of the protocol of Tautz and Pfeifle (1989) using 55°C as hybridization temperature. For examination, ovaries were mounted in araldite.

4.3. Antibody staining

Antibody stainings against the activated form of MAPK were performed using an anti-activated MAPK polyclonal rabbit antiserum from Promega. The anti-Gurken antiserum was raised in rabbits against a *N*-terminal fragment of the Gurken protein including residues 110 to 174 as a GST fusion (Neumann-Silberberg and Schüpbach, 1993). The antiserum was affinity purified against the same protein bound to MiniLeak matrix (Kementec) and extensively preabsorbed against *grk*^{2B6} homozygous ovaries. Bound antibody was detected using alkaline phosphatase coupled goat-anti-rabbit serum (Dianova) and BCIP/NBT (Boehringer).

Antibody staining and sectioning of the embedded ovaries were performed as described by Roth et al. (1995) with the following modifications: ovaries were fixed in 4% para-formaldehyde and heptane including 0.5% NP-40 for 20 min. After washing in buffer containing 0.1% BSA, the ovaries were pretreated with 0.3% H_2O_2 in MeOH for 20 min and rehydrated in MeOH/PBST. For the MAPK stainings a short treatment with Proteinase K was performed in order to improve the penetration of the MAPK antibody.

4.4. Cloning of *grk* from *Drosophila* virilis

Phages containing *grk* sequences were isolated from a EMBL3 *D. virilis* genomic library (Paul Schedl) by low stringency hybridization with a ^{32}P labeled *D. melanogaster grk* probe. Insert DNA was prepared according to the protocols in Sambrook et al. (1989). The position of the introns was determined by sequencing of the *grk* coding region from cDNA obtained through reverse transcription PCR (Superscript, Promega) of polyA mRNA isolated from dissected *D. virilis* ovaries with mRNAdirect (Dynal). Sequencing of the clones was performed using an ALFexpress automated sequencer (Pharmacia) and dye primer cycle sequencing chemistry (Amersham).

5. Note added in proof

After submission of our manuscript, a paper addressing related questions has appeared in Cell (Wasserman, J., Freeman, M., 1998. Cell 95, 355–364). The authors demonstrate the existence of a second round of DER activation during follicle cell patterning using somatic clones of *spitz*, *argos* and *rhomboid* mutations.

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